THE DIRECT DETERMINATION OF CREATINE IN PATHOLOGICAL URINE

BY

G. S. WALPOLE, D.Sc., F.I.C.

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By G. STANLEY WALPOLE, B.Sc., F.I.C.

(*From the Wellcome Physiological Research Laboratories, Herne Hill, S.E.*)

THE investigation by Harden[1] into the nature of Vosges and Proskauer's reaction[2] led to the discovery that acetyl methyl carbinol and air or else diacetyl were necessary factors, and also that a substance containing a certain atomic configuration, such as arginine or creatine, must be present. Creatinine does not contain the necessary grouping and this suggested the method for the direct determination of creatine in urine that is the subject of the present communication.

The estimation of creatine in urine is based on the fact that creatine gives a pink colouration with diacetyl in alkaline solution. The depth of the colour produced by the reaction is influenced so much by a very large number of apparently insignificant factors that it was found necessary to adopt certain standard conditions. Test-tubes size $6'' \times \frac{3}{4}''$ are employed, and in any examination in which this reaction is used there are taken in each tube 2 c.c. of a saturated sodium carbonate solution, 2 c.c. of the solution examined, the requisite number of drops of diacetyl solution and water up to a 6 c.c. mark. The tubes to be compared are then stood vertically in boiling water for one minute, allowed to stand in a test-tube rack for half an hour and examined, after filtration if necessary, in special glass tubes with bottoms of optically worked glass.

At the ordinary temperature, the reaction when the solution examined contains creatine proceeds very slowly. The depth of colour passes through a maximum after about twelve hours and then

[1] Harden. *Proc. Roy. Soc.* B. LXXVII. p. 421. 1906.

[2] Vosges and Proskauer. *Ztsch. f. Hyg.* XXVIII. p. 20. 1898.

diminishes in intensity. The rapidity with which these phases follow one another is increased very greatly by warming, and I have found the conditions given to be the most suited to the present problem.

Preparation of the diacetyl solution. The synthesis from methyl-acetoacetic ester described by v. Pechmann[1] is given in Beilstein's *Handbuch* and can readily be performed in a few hours. I have found it convenient to convert the diacetyl at once into the dioxime and prepare fresh diacetyl solution when required by simply distilling a little of this substance with dilute sulphuric acid. If to 1 gram of oxime 100 c.c. of water be added together with 20 c.c. of strong sulphuric acid and the whole distilled gently till 50 c.c. of distillate are collected there will be obtained the solution which throughout this communication is referred to as "a fresh solution of diacetyl." Dimethyl glyoxime is listed by dealers in pure chemicals so that the preliminary synthesis of diacetyl can be avoided, and diacetyl can also be obtained.

Technique. The ammonium salts and probably some other substances in urine inhibit to a certain extent the production of the pink colour from creatine and diacetyl. It was found, however, that although the colour produced under standard conditions by 2 mgm. of creatine in 10 c.c. of urine was not nearly so intense as that produced by 2 mgm. of creatine in 10 c.c. of water, yet if two samples of urine were compared after adding equal qualities of creatine to each, the increase of colour due to the creatine content was the same in both cases. This was tested repeatedly and it was found in practice that this, subject to certain restrictions given below, worked out very well.

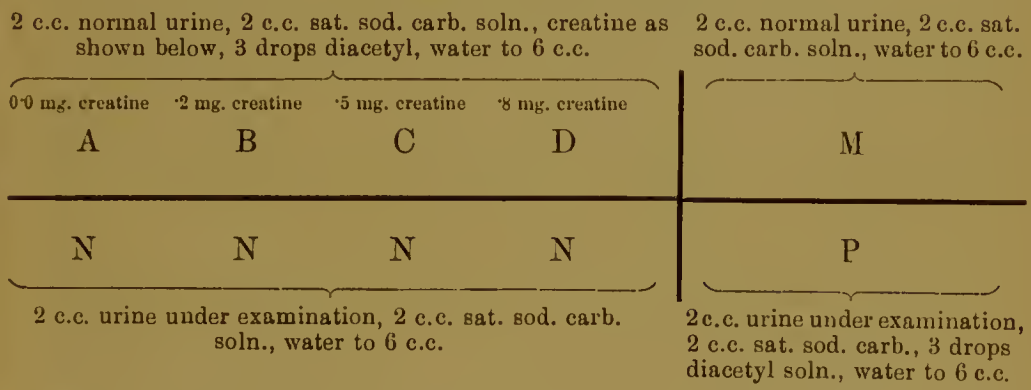
The method is to take creatine-free normal urine and add known amounts of creatine and, by trial, discover what amount gives the same colour with diacetyl in normal urine that the unknown quantity does in the urine under investigation. It is also arranged that an exact compensation is made for the difference in colour between the normal urine and the urine examined. No method involving the measurement of depth of tint is applicable for reasons which are given in the footnote at the bottom of p. 304.

They are all heated and set to cool as has been described. The reactions in those tubes containing diacetyl are now considered complete. The contents of A and N are mixed and so also those of B and N: C and N: D and N: and P and M, and filtered if they are not already perfectly clear. This filtration is important as any precipitate masks the true depth of

[1] v. Pechmann. *Ber.* xxiii. p. 2427. 1890.

colour of the solution. The filtrates are collected in test-tubes of even bore, or better in Nesslerising glasses and the particular one PM compared with the others. The comparison gives directly the creatine content of the urine investigated. Should PM be darker in colour than ON it contains more than ·8 mgm. in 2 c.c. and a fresh determination must be made with the urine suitably diluted. The dark red colour obtained when the creatine in PM is more than ·8 mgm. (4 mgm. in 10 c.c. of urine) cannot be readily compared with DN, neither do the results obtained with such concentrated creatine solutions have much value. If the contents of the two tubes which match—say CN and PM—be considered, it will be seen that each contains the same amount of urinary pigment and that in each case the reaction took place in urine. It is, therefore, concluded that the creatine present in both cases is the same, *i.e.* 2 c.c. of the urine examined contains the same amount as that in tube C, which is ·5 mgm.

Ten test-tubes are placed in a rack and filled as indicated in the diagram.



If the determination has been correctly performed it will be seen that

1. The colours of the tubes A, B, C, D after standing and cooling are progressively darker.

2. The contrast between A and M in colour is the more marked the less fresh is the specimen of diacetyl employed. It should be much less than and readily distinguishable from the contrast between B and A.

In the case where the urine under examination and the normal urine are of the same colour, or nearly the same colour, the precautions to correct for this difference need not be applied and only the tubes A, B, C, D and P need be prepared. A comparison of P with the others gives the result.

Colour due to polymerisation of diacetyl. Considerable difficulty is encountered in that diacetyl itself polymerises very rapidly in alkaline

solutions, forming a brown pigment, which renders the quantitative observation of the pink colour due to its reaction with creatine less accurate. Excess of diacetyl is, therefore, to be avoided and the total colour in any case is due to

1. the colour of the urinary pigment under the standard conditions of the experiments;
2. the colour due to the diacetyl-creatine reaction;
3. the colour due to the polymerisation of the excess of diacetyl.

Of the fresh solution three drops are sufficient to give a graded increasing depth of pink colour in tubes containing from ·2 to ·8 mgm. of creatine under the standard conditions laid down.

The colour due to polymerisation of the excess will scarcely be noticed unless an endeavour is made to measure its depth by a colorimeter of any kind[1]. If the diacetyl solution is kept it undergoes some change, probably a condensation, and the depth of the pink colour given with a certain amount of creatine under standard conditions diminishes. As the solution gets older, therefore, it is necessary to employ more and more of it, and as it retains its property of turning brown with alkali unimpaired, a time ultimately arrives when the brown pigment becomes really troublesome and it is found advisable to commence again with a fresh preparation. One sample can be used conveniently for a month and for rough work for much longer if the operator is experienced in the reaction.

The nature and amount of the alkali used. A number of trials were made before it was decided to use 2 c.c. of saturated sodium carbonate solution in each tube. Stronger alkali converted some of the urinary creatinine into creatine during the process of warming for one minute and subsequently standing to cool; on the other hand, the pink colour is not developed if the liquid is not sufficiently alkaline. It was decided to use a strong sodium carbonate solution instead of a weak caustic soda solution, because the ammonium salts in the former case exhibited to a lesser extent the inhibiting action described below.

Effect of ammonium salts in urine. It was observed unmistakably that the brown colour due to diacetyl polymerisation and the colour due

[1] When such an attempt was made it was at once seen that the change of colour from tube to tube was not only a change of intensity but also of tint as opposed to colour density. A true colorimetric process of which the Folin method is an admirable example is based on the assumption that throughout the solutions examined, tint, as opposed to depth of colour, is constant: it is clearly impossible to determine the equality or inequality of the depth of colour of the contents of two tubes when the tints in the two cases are different.

to the creatine-diacetyl reaction were less when the creatine was contained in urine than when it was contained in water. This is apparently due to two causes. The presence of ammonium salts interferes with the reaction, and experiments were made in which the free ammonia in the urines examined was determined by Folin's method[1]. Corresponding amounts of ammonia were added to some determinations of creatine in water, using, as usual, 2 c.c. of saturated sodium carbonate solution, diacetyl and water to 6 c.c. It was found in each case that the colour obtained was materially diminished by the ammonia added. To a number of samples of normal urine giving no indication, by this method, that creatine was present, known amounts of creatine were added. They were compared one with another by the method given, and it was found roughly that those with the highest free ammonia content gave the least increase of colour with diacetyl and alkali under standard conditions, and *vice-versa*. The accord was not perfect, and this led me to seek for the disturbing factor.

From some normal urine phosphates were removed by baryta, and free ammonia by adding sodium carbonate and the passing air for some hours[2]. It was found that creatine added to the resulting solution still gave slightly less colour than it did in water. The second substance having the same effect as free ammonia is, together with urinary pigment, removed from urine by animal charcoal. It is not extracted by ether, and in this respect differs from the substance recently found in urine by Buckmaster[3], which inhibits the pseudo-peroxydase reaction for blood.

Removal of albumen. Protein gives the pink colour with diacetyl and alkali. To remove it I found trichloracetic acid satisfactory. It does not effect the reaction except in so far as it neutralises the sodium carbonate present. It should be added equally, of course, to all the tubes to which diacetyl is added.

Comparison of results obtained by the Folin method and the diacetyl method above described. The method universally adopted is that of Folin[4], in which the total creatinine before and after acid hydrolysis is estimated by the technique elaborated by him. Although this procedure postulates several debateable points, and is a determination involving the small difference of two measured quantities, it proves in practice to be satisfactory. The original method described by Folin

[1] Folin. *Ztsch. f. Physiol. Chem.* XLI. p. 223. 1904.
[2] Matthew Steel. *Journ. Biol. Chem.* VIII. p. 365. 1910.
[3] Buckmaster. *Proc. Physiol. Soc.* p. xi. 1908 (This *Journ.* XXXVII.).
[4] *loc. cit.*

has been altered very little by subsequent observers. E. Mellanby[1] advocates plotting a curve once and for all, thus obviating the necessity of repeating the determination should the readings fall outside the range 5—12 mm. as Folin recommends. In connection with this curve it would appear at first sight that the accuracy of the determination is much greater with dilute than with concentrated solutions, but the fact that when the solution is more coloured the colorimeter can be adjusted with correspondingly greater accuracy leaves the order of magnitude of the error in reading constant over a very considerable range. The personal factor is a very great one in this method and a recent criticism by Taylor[2], advocating a standard illumination, is very much to the point.

The results given are for a number of pathological urines kindly supplied by Dr E. Mellanby, whose creatine determinations by the Folin method I have his permission to quote.

TABLE I.

Sample No.	Creatine mg. in 10 c.c. by Folin's method	Creatine mg. in 10 c.c. by diacetyl method
1	1·2	2·1
2	5·5	5·1
3	2·3	2·5
4	1·2	1·1
5	4·0	5·0
6	2·0	3·0
7	4·5	3·0
8	0·8	1·5
9	2·7	3·0
10	2·7	3·5
11	5·2	3·5
12	4·4	greater than 6·0
13	5·4	5·0
14	3·4	4·5
15	3·5	3·0

Such differences as exist do not point to any consistently higher values being given by one method than by the other. Further experiments are required to show which is the more accurate method of creatine determination. The absolute accuracy of the diacetyl method is possibly of the same order as that of the method of Folin. It might be considerably increased if the free ammonia of the urines was in each case determined and made up to a constant quantity.

[1] E. Mellanby. This *Journal*, xxxvi. p. 453.

[2] A. E. Taylor. *Journ. of Biol. Chem.* pp. 19–20. 1911.

Substances which may possibly occur in pathological urines which give the reaction. The reaction with diacetyl and alkali is given not only by creatine, but also by arginine and any protein containing arginine[1]. How much arginine is required to give, under the conditions of experiment adopted, the same colour as 1 mgm. of creatine has not been determined. An attempt was made to compare the creatine content in urine before and after acid hydrolysis, but the deep pigmentation of the urine when heated with acid made this impossible. W. H. Thompson[2] failed to obtain arginine in the urines which he examined, and I think it probable that any investigation of the occurrence in urine of a substance other than creatine containing the particular atomic linking to which the reaction is ascribed, will be considerably facilitated by the comparison of the Folin and the diacetyl estimations. Arginine does not reduce alkaline picrate solution. It may possibly happen that further work on this subject will demonstrate how far the differences in these creatine determinations are due to experimental error and to what extent they may be used to show in urine the presence of other substances containing in their structure the particular atomic linkings upon which the diacetyl reaction depends.

A Clinical Method. For an approximate determination of the amount of creatine in a sample of urine I would recommend the following method.

Four test-tubes are taken and filled as indicated:

(1)	(2)	(3)	(4)
2 c.c. sat. sod. carb. soln.	2 c.c. sat. sod. carb. soln.	2 c.c. sat. sod. carb. soln.	2 c.c. sat. sod. carb. soln.
2 c.c. normal urine.	2 c.c. normal urine.	2 c.c. urine under examination.	2 c.c. urine under examination.
3 drops fresh diacetyl soln.	·4 mg. creatine in soln.	Water to 6 c.c.	3 drops fresh diacetyl soln.
Water to 6 c.c.	3 drops fresh diacetyl soln.		Water to 6 c.c.
	Water to 6 c.c.		

All are put into boiling water for one minute and then examined. It is seen that (1) is scarcely altered, (2) has developed a bright pink tinge, (3) is not changed, and on (4) the diagnosis depends. If it is seen, however, that (4) does not show the pink tinge that (2) does when compared with (1), then it can be concluded that there is no creatine present. The development of a pink tinge in (4) can be taken as indicating the presence of creatine in amount which may be judged by

[1] Harden and Norris. This *Journal*.

[2] W. H. Thompson. This *Journal*, XXXIII. p. 106.

observing tube (2), which is known to owe its colour to 2 mgm. in 10 c.c. of urine. Diacetyl has been placed in (1) in order that the increase of colour, due to its polymerisation, may be observed and may not be mistaken for the distinct pink tinge of the diacetyl creatine reaction.

Summary.

The pink colour given in alkaline solution by creatine but not by creatinine when a trace of diacetyl is added has been utilised for the quantitative estimation of creatine in urine.

The method is simple and rapid and it has been found possible after adding creatine to urine to measure with a fair degree of accuracy the amount added. Comparative results of a number of pathological urines in which the creatine was estimated by both the Folin and the diacetyl methods are also given.